Examining the basis of isoniazid tolerance in non-replicating *Mycobacterium tuberculosis* using transcriptional profiling

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**Defined key terms**: 5-10

***Mycobacterium tuberculosis*:** Causative bacterial agent of tuberculosis, responsible for 1.8 million deaths per year, with an estimated 2 billion people infected worldwide.

**Isoniazid:** Front line anti-tuberculosis drug targeting fatty acid synthesis (FASII) and affecting the mycobacterial cell wall; responsible for the majority of mycobacterial killing within the first 2 days of treatment with standard regimen.

**Persistence:** Bacilli capable of causing active disease that survive prolonged chemotherapy, resulting in lengthy and expensive treatment for tuberculosis.

**Phenotypic drug tolerance:** Mechanisms by which genetically susceptible bacilli are refractory to killing with anti-microbial agents. Tolerance may be dependent on pre-existing sub-populations of physiologically distinct bacilli, or may be initiated by changes in the microenvironment or drug exposure.

**Transcriptional profiling by microarray:** Exploring the global responses of bacilli to the surrounding microenvironment by simultaneously quantifying mRNA abundance for all defined *M.tuberculosis* genes.

**Wayne model:** An *in vitro* model where primarily oxygen is limited to mimic *M.tuberculosis* non-replicating persistence.

## Abstract

**Background:** Understanding how growth state influences *M.tuberculosis* responses to antibiotic exposure provides a window into drug action during patient chemotherapy. Here, we describe the transcriptional programs mediated by isoniazid (INH) during the transition from log phase to non-replicating bacilli, from INH-sensitive to INH-tolerant bacilli respectively, using the Wayne model. **Results and Conclusions:** INH treatment did not elicit a transcriptional response from non-replicating bacteria under microaerophilic conditions (NRP2), unlike the induction of a robust and well-characterized INH signature in log phase bacilli. The differential regulation (between drug-free NRP2 and log phase bacilli) of genes directly implicated in INH resistance could not account for the abrogation of INH killing in non-growing bacilli. Thus, factors affecting the requirement for mycolic acids and the redox status of bacilli are likely responsible for the reduction in INH efficacy. We speculate on additional mechanisms revealed by transcriptome analysis that might account for INH tolerance.

## Introduction

While effective at resolving active *Mycobacterium tuberculosis* disease, current TB chemotherapy requires multiple drugs to be taken over six months to clear infection of genotypically drug-sensitive bacilli. This long treatment period exerts huge demands on healthcare infrastructure and finances, and risks the emergence of drug resistance through poor patient compliance. The search for a more effective shortened drug regimen is complicated by the presence of persistent organisms, likely phenotypically drug tolerant bacilli, that need prolonged drug treatment to clear [(1)] [(2)].

Phenotypic tolerance, unlike genetically defined resistance, is a transient bacterial state in which many antibiotics are ineffective. Tolerance is likely dependent upon pre-existing sub-populations of genetically susceptible bacteria in a slow/non-multiplying state that are refractory to drug action [(3)]. This small fraction of phenotypically distinct bacteria enable bacterial populations to survive dramatic changes in the environment [(4)]. In addition to stochastic mechanisms that create sub-populations of non-growing cells in a phenotypically heterogeneous bacterial community, recent evidence suggests that tolerant bacterial sub-populations may be generated after antibiotic stress [(5)]. In TB clinical settings, phenotypically tolerant bacilli are likely to be responsible for the persistence of *M.tuberculosis* bacilli through the drug treatment period [(6)]. We speculate that phenotypically tolerant bacilli in patients may exist as a result of: i) stochastic processes; ii) through exposure of bacilli to multiple microenvironments imposing individual constraints on growth that may be temporally and spatially separated; and iii) through prolonged antibiotic

stress. There is sparse information defining sub-populations of *M.tuberculosis*, but both clinical data and the demonstration of sub-populations from *in vitro* culture and animal models [7] [8] indicate that sub-populations exist. Further *in vivo* evidence is provided by quantitative variations in lipid inclusion body staining of human sputum-derived bacilli [9], and the observation that bacilli in different locations of human lung tissues display unique gene expression patterns [10]. Furthermore, murine models of chronic infection show increased tolerance to isoniazid, suggesting slow growth rates are associated with reduced drug efficacy [11].

Isoniazid (INH) is a front-line drug in TB treatment and prophylaxis. Bactericidal activity during the first 2 days of TB treatment is almost entirely due to INH eliminating around 90% of bacilli [12]. However, minimal killing is observed after exposure of non-replicating *M.tuberculosis* bacilli to INH *in vitro* [13], so it is unlikely that INH contributes to the clearance of persistent bacilli in patients during the extended treatment phase. INH is a pro-drug that enters the cell by passive diffusion where it is converted by a *katG*-encoding catalase-peroxidase to an active INH-NAD adduct; reviewed in [14]. The primary target of INH-NAD is InhA, an enoyl-ACP reductase, part of the FASII cycle, that is required for mycolic acid synthesis and maintenance of the mycobacterial cell wall structure [15] [16]. The INH-NAD(P) adduct(s) are likely to inhibit multiple secondary targets, such as the NADPH-dependent dihydrofolate reductase, that may also contribute to INH-mediated killing [17]. Resistance to INH is most frequently determined by mutations in *katG*, inactivating the catalase-peroxidase required for INH pro-drug conversion. Mutations in the promoter of *inhA*, resulting in the

over-expression of the target (*inhA*) or affecting the InhA active site also confer resistance [18]. Mutations in a number of other genes have been identified in INH-resistant *M.tuberculosis* strains [19]. However, approximately 22-29% of INH-resistant strains do not possess mutations in genes known to affect INH-resistance [20] [21], indicating that there may be unexplored mechanisms that influence INH efficacy in *M.tuberculosis*.

Transcriptional profiling by microarray analysis has been used extensively to define the *M.tuberculosis* genome-wide responses to antibiotic exposure (classifying drug action and identifying possible resistance mechanisms) [22] [23] [24] and fluctuations in environmental factors such as limited oxygen [25] [26] [27] or nutrient starvation [28] [29]; reviewed in [30]. In one such model of mycobacterial persistence developed by Wayne and colleagues [31], oxygen levels are gradually reduced by stirring liquid cultures in sealed tubes. Under these conditions, bacilli enter a physiological state known as non-replicating persistence (NRP), which is characterized by two distinct stages. The first, NRP1, occurs in microaerophilic conditions when the concentration of oxygen in the medium descends to 1%. During this phase, bacilli shut down DNA synthesis and cell division, restrict biosynthetic activity, and use alternative energy sources to stabilize and protect essential cell components. The second NRP2 stage, where general metabolism is reduced further and replication ceases, occurs in anaerobic conditions when the dissolved oxygen concentration drops below 0.06% [13]. Non-replicating bacteria may become tolerant to antibiotic exposure [32] [33]; bacilli in NRP2 exhibit some definite but reduced killing with rifampicin and are highly tolerant to isoniazid and

ciprofloxacin [31]. Furthermore the absence of an isoniazid-inducible transcriptional signature has been used to characterize *in vitro* and *in vivo M.tb* dormant states [34].

In an attempt to understand why non-replicating bacilli are refractory to killing by INH, and thus to identify putative INH-tolerance mechanisms mediated by changing transcript abundance, we defined the transcriptional responses to INH exposure in log phase and Wayne model NRP2 bacilli. Having contrasted the changing pattern of gene expression through the transition from log phase to NRP2 with the INH-induced transcriptome signature, we also discuss INH tolerance in non-growing populations and consider mechanisms that may influence drug tolerance.

## Experimental

### Aerobic log-phase and Wayne model cultures

Seed cultures of *Mycobacterium tuberculosis* H37Rv were incubated in 10 ml Dubos liquid medium (Difco) supplemented with Dubos medium albumin, with shaking at 37ºC for 6 days to an optical density 580 nm of 0.4. Both aerobic and non-replicating persistence (NRP) cultures were set up in glass tubes (125 by 20 mm) with screw-top lids containing 1.5 mm magnetic stir bars, and stirred at 120 rpm as characterized by Wayne [35]. Log phase bacilli were incubated for 3 days in 10 ml Dubos medium in tubes with loosened caps. The Wayne model cultures were incubated for 6 days (NRP1) or 21 days (NRP2) in 17 ml Dubos medium in tubes with sealed lids (with a resulting head-space ratio of 0.5). Growth was monitored by measuring optical density every 24h. RNA was isolated from aerobic log-phase and Wayne model cultures on two independent occasions.

### Isoniazid treatment

Cultures were treated with 0.2 μg/ml isoniazid (INH) final concentration (from 100 μg/ml stock solution, Sigma) at days 6 and 21 in the Wayne model (for NRP1 and NRP2 respectively), and at day 3 in log phase aerobic cultures. An equivalent volume of sterile water was added to drug-free carrier control samples. Mycobacterial RNA was extracted after exposure to INH for 4h. Prior to addition, syringes containing INH (or sterile water) were pre-incubated in an anaerobic cabinet overnight, to eliminate oxygen from the solutions and prevent the reintroduction of dissolved oxygen to the NRP cultures.

**Mycobacterial RNA isolation**

RNA was extracted using the GTC/TRIzol method [36] [37]; at each timepoint the culture volume (8 tubes per condition) was added to 4 volumes of 5 M guanidine thiocyanate solution, and the bacteria harvested by centrifugation. The bacilli were lysed in TRIzol using a reciprocal shaker, and the nucleic acid extracted with chloroform, before isopropanol precipitation. The RNA samples were purified and DNase I treated on RNeasy columns (Qiagen). Total RNA concentration was determined by spectrophotometry (NanoDrop ND-1000 3.1, NanoDrop Technologies), and size distribution assessed using an Agilent 2100 Bioanalyser (Agilent Technologies).

**Microarray hybridization**

Whole-genome microarrays generated by the Bacterial Microarray Group at St. George's (ArrayExpress accession number A-BUGS-23 [101]) were hybridized as described previously [38] [37]. Briefly, 5 μg cDNA derived from each RNA sample and 2 μg *M.tb* H37Rv genomic DNA were labeled with Cy5-dCTP and Cy3-dCTP respectively. RNA extracted from two biological replicates were hybridized in triplicate, resulting in 6 hybridizations per condition. Microarrays were scanned at 532 nm and 635 nm corresponding to Cy3 and Cy5 excitation maxima using the Affymetrix 428™ Array Scanner (MWG). Comparative spot intensities from the images were calculated using Imagene 5.5 (BioDiscovery), and the data imported into GeneSpring GX 7.3.1 (Agilent Technologies) for analysis. The data were normalized to the 50th percentile of all genes detected to be present, and filtered to include genes flagged to be present in 80% of the arrays.

**Transcriptional profiling analysis**

Genes significantly differentially expressed between growth states or after isoniazid treatment were identified using a t-test with Benjamini and Hochberg multiple testing correction ($p<0.05$), and a minimum fold change of 1.5. Genes were hierarchically clustered using Cluster and the results displayed using Treeview [39]. The hypergeometric distribution was used to determine if previously identified gene expression signatures, or genomic functional categories were significantly enriched in each comparison [40]. Significantly differentially expressed genes are detailed further in Supplementary Tables 1 and 2. Fully annotated microarray data from this study have been deposited in BμG@Sbase (accession number: E-BUGS-104 [102]) and ArrayExpress (accession number: E-BUGS-104).

**Quantitative RT-PCR**

Primers and probes for *cyd*A, *efp*A, *icl*, *inh*A, *ini*A, *kas*A, *kat*G, *narX*, *ndh* and *tgs*1 were designed using the Primer Express 1.0 software (Applied Biosystems), and are detailed further in Supplementary Table 3. All probes were labeled with FAM (5-carboxyfluoroscein) at the 5', and TAMRA (N,N,M',N'-tetramethyl-6-carboxyrhodamine) at the 3'. An internal control, s*ig*A (labeled with VIC), was used to normalize mRNA levels. Mycobacterial RNA (5 μg) was reverse transcribed in a total volume of 25 μl using random primers (Invitrogen) according to the manufacturer's instructions. 1 μl of template cDNA was used for qRT-PCR with AmpliTaq Gold polymerase, alongside no template and no RT negative controls. qRT-PCR were performed using an ABI PRISM 7700

Sequence detector system (Applied Biosystems), amplification conditions 95ºC for 10 min followed by 50 cycles at 95ºC for 15s and 60ºC for 60s. Each reaction was performed in triplicate. The ΔΔCt method [41] was used to determine changes in relative gene expression.

## Results and Discussion

### Gene expression programs illustrate the transition from log phase to non-replicating persistence

To examine the relationship between growth rate and phenotypic drug tolerance we used genome-wide microarray RNA profiling and qRT-PCR to map the transcriptional status of *M.tuberculosis* with and without exposure to isoniazid (INH) in log phase and in the Wayne model of non-replicating persistence (NRP). We hypothesize that transcriptome patterns may reveal mechanisms that explain INH tolerance, such as changes in expression levels for genes involved in drug efflux, alteration in target levels (*inhA*) or reduced *katG* expression (activation of drug). We first compared drug-free NRP bacilli to log phase bacilli (drug-free NRP2 *vs.* drug-free log phase) to describe the changes in gene expression that occur as oxygen and nutrients become limiting, as defined by Wayne and Hayes [(31)].

As mycobacterial growth rate slows and then plateaus, 944 genes were significantly differentially expressed in NRP1 (323 induced, 621 repressed) and 1451 genes differentially regulated in NRP2 (649 induced, 802 repressed) compared to aerobic log phase growth (Supplementary Table 1). As expected, there were highly significant overlaps in gene expression patterns with previous studies characterizing the transcriptional response of bacilli to stationary phase growth [(26)], oxygen limitation [(25)], nutrient starvation [(28)] and the Wayne model itself [(42)], with hypergeometric p-values for enrichment of NRP2-induced genes of $7.17 \times 10^{-38}$, $3.44 \times 10^{-18}$, $4.23 \times 10^{-16}$ and $9.36 \times 10^{-31}$ respectively. Genes with functional classifications [(43)] such as aerobic respiration, ribosomal protein

synthesis and ATP-proton motive force were repressed in NRP2 compared to aerobic axenic growth (hypergeometric p-values $5.14x10^{-8}$, $8.52x10^{-6}$ and $4.34x10^{-5}$ respectively). Conversely, the *dosR* [44] (hypoxia and NO responsive dormancy regulon), *mprA* [45] (2-component response regulator) and *kstR* [46] (cholesterol metabolism) regulons were significantly induced in NRP2 compared to log phase growth (hypergeometric p-values $5.85x10^{-28}$, $6.10x10^{-5}$ and $1.20x10^{-4}$ respectively); as were the alternative sigma factors *sigB*, *sigE*, *sigF*, *sigG* and the regulator of the stringent response, *relA*. The transition from rapidly multiplying to non-replicating bacilli in this Wayne model of dormancy mirrors previously identified transcriptional changes reflecting a change in respiratory state from aerobic to anaerobic as oxygen becomes limiting, a switch to lipid-based carbon sources as nutrients become scarce, and a modification of cell wall composition and a slowing of metabolism as growth terminates [27] [47].

**Non-replicating bacilli to do not mount a transcriptional response to isoniazid**

We then asked whether the transcriptional responses to INH would differ between these two physiologically distinct populations of bacilli. The hypothesis being that drug-induced responses reveal genes involved in potential resistance mechanisms or compensatory responses that reduce the effect of the drug and hence may underpin tolerance. We defined genes differentially expressed after 4 h exposure to 0.2 μg/ml isoniazid in log phase and NRP2 bacilli (in INH-treated log phase *vs.* drug-free log phase, and INH-treated NRP2 *vs.* drug-free NRP2 comparisons).

In rapidly multiplying log phase bacilli, 100 genes were significantly differentially regulated after 4h INH treatment; with 71 genes induced and 29 genes repressed relative to drug-free log phase bacilli (Supplementary Table 2). There was a high degree of correlation between INH-responsive genes identified in this investigation and previous studies examining INH-mediated transcriptional programs; hypergeometric p-values [48] $2.15x10^{-24}$; [22] $5.79x10^{-16}$; [23] $3.54x10^{-12}$; and [49] $5.20x10^{-10}$. Genes indicative of cell wall inhibitors such as *accA3*, *accD4*, *ahpC*, *ahpD*, *efpA*, *fadD32*, *fbpC*, *htdX, iniA*, *iniB*, *lprJ*, *pks16* and *Rv3717* were significantly induced [23]. Contrastingly, no genes were significantly differentially expressed after INH treatment of NRP2 bacilli relative to drug-free NRP2 bacilli. To ensure that the absence of differentially regulated genes after INH treatment in NRP2 bacilli was not an artifact of significance testing, we plotted the expression pattern of log phase INH-responsive genes in both log phase and NRP conditions (Figure 1a/b). Figure 1a shows a heat map of 6 replicates of all genes significantly up-regulated by INH in log phase bacilli and the gradual diminution of transcriptional response as transition proceeds through NRP1 towards NRP2. This confirmed that genes comprising the INH transcriptional signature of log phase bacilli were not differentially regulated after INH treatment in NRP2 bacilli. These observations were verified by quantitative RT-PCR using a panel of genes selected to be indicative of mycobacterial growth state and implicated in isoniazid resistance (Figure 2). Thus, increased tolerance of NRP2 bacilli to isoniazid seems not to be dependent on the initiation of a transcriptional program after exposure to INH that renders the drug ineffective.

The absence of a transcriptional response to isoniazid indicates no activity of the drug in NRP2 bacilli. This could be explained in several ways: i) INH has not entered the cell due to alterations in cell wall structure, or has been exported rapidly; ii) INH is not converted to the toxic product by KatG, or there is reduced availability of NAD to form the INH-NAD adduct; or iii) the enzyme target(s) of INH-NAD outnumber the toxic adducts generated, or the inhibition of pathways affected by INH are no longer functionally significant for bacilli in NRP2. We have utilized the gene expression signatures of NRP2 and INH as a framework to explore these hypotheses further.

**Differential regulation of genes identified to harbor mutations in INH-resistant *M.tuberculosis* strains do not explain INH tolerance in NRP2**

Following the hypothesis that differences in transcript abundance between log phase and NRP2 bacilli might influence INH tolerance; we asked whether the differential regulation of genes associated with INH resistance might explain INH tolerance in NRP2 bacilli. On the assumption that differential expression of these genes (drug-free NRP2 *vs.* drug-free log phase) may relate to changes in protein (or drug) activity resulting in tolerance, even though these genes may not be directly involved in the mechanism of drug action. We therefore compared the expression profiles of 18 genes with mutations in INH drug-resistant strains, collated on the Tuberculosis Drug Resistance Mutation Database [(19)], and present in the filtered microarray dataset used in this study (Figure 3).

Mutations that result in the over-expression of *inhA* (increased target) lead to INH resistance by overwhelming the active INH-NAD adduct present in exposed bacilli [14], however both *inhA* and the upstream gene *fabG1* were repressed in NRP2 relative to log phase bacilli. An alternative mechanism of drug resistance might be caused by the increased activity of NAT (arylamine N-acetyltransferase), which inactivates INH by acetylation leading to resistance. However the induction of *nat* was not observed in NRP2 compared to log phase bacilli. The catalase-peroxidase activity encoded by *katG* is required for the formation of the INH-NAD adduct; mutations in this gene result in INH resistance. Therefore, a down-regulation of *katG* could be hypothesized to increase INH tolerance. However, *katG* expression was induced in NRP2 relative to log phase bacilli, which might be predicted to increase rather than decrease the susceptibility of NRP2 bacilli to killing by INH. Conversely, the activity of this catalase-peroxidase is reduced at low oxygen levels [50], which might result in the formation of less INH-NAD adduct and thus INH tolerance. Notably, this reduction of KatG activity in hypoxic conditions might also be predicted to result, through compensatory feedback mechanisms, in the induction of *katG* gene expression in NRP2 compared to log phase bacilli, which was observed. The repression of *ndh*, leading to a reduction in NDH-attributed NADH dehydrogenase activity and an accumulation of NADH that acts as a competitive inhibitor with INH-NAD for INHA binding [51], increasing drug tolerance, was not evident.

Genes identified as potentially influencing INH activity because they are highly up-regulated after INH treatment [52] [22] (such as *fbpC*, *iniBAC*, *Rv1592c*,

*Rv1772* and *efpA*), were not induced in NRP2 compared to log phase growth as might be the case if these gene products contributed to INH tolerance in NRP2 (Figure 3). Interestingly, *fadE24*, a probable acyl-CoA dehydrogenase likely involved in the β-oxidation of fatty acids that is induced after INH exposure, was marginally induced in NRP2 relative to log phase bacilli. It has been previously proposed that the induction of genes involved in lipid degradation may be responsible for recycling fatty acids that accumulate after exposure to INH and the inhibition of the FASII cycle [22]. The differential regulation of genes encoding efflux pumps may modify INH entry and exit kinetics affecting killing efficacy: *efpA*, *mmr*, *Rv1819c*, *Rv2459* and *Rv3728* have been demonstrated to be induced by INH in multi-drug resistant strains [53]. However none of these genes were significantly induced in NRP2 relative to log phase growth. Of the genes assigned by Cole *at al*. [43] to the functional category III.A.6 efflux genes, 3 were induced in NRP2 *Rv1250* (a probable drug-transport integral membrane protein, member of the major facilitator superfamily), *Rv1634* (a possible drug efflux protein, also member of the MFS family) and *Rv2209* (a conserved integral membrane protein); 4 genes were repressed relative to log phase bacilli (*drrB*, *drrC*, *emrB* and *Rv2459*). This may be a possible explanation for increased efflux-based tolerance, but little is known about the specificity of each transporter. The expression profiles of a subset of these genes were verified by qRT-PCR (Figure 4); this showed excellent correlation between ratios generated from microarray and qRT-PCR techniques.

**Complex transcriptional responses reflecting the changing physiological state of bacilli in NRP2 likely affect INH efficacy**

Unlike mutations to single genes that affect the primary target of INH or its modification into an active molecule that result in INH resistance, adaptations involving the differential expression of many genes involved in respiratory and metabolic pathways may influence INH tolerance in NRP2. For example, the maintenance of redox potential in NRP2 bacilli is very different to log phase aerobically respiring bacilli. This may prohibit the conversion of INH to INH-NAD adduct (akin to the inactivation of KatG). Cytochrome P450 genes and their putative redox partners [54] were differentially regulated in NRP2 relative to log phase; with *cyp51*, *cyp124*, *cyp125*, *cyp130*, *cyp132*, *fdxA*, *fdxC*, *fdxD* and *fprB*, *fprD* induced, and *cyp121*, *cyp135A1*, *cyp136*, *cyp140*, *cyp141*, *cyp143* repressed. Additionally, the requirement for cytochrome F420 may differ [47] with the NADPH-dependent glucose-6-phosphate dehydrogenases (*zwf1* and *zwf2*) down-regulated, and F420-requiring glucose-6-phosphate dehydrogenases (encoded by *fgd1* and *fgd2*) induced in NRP2. Mutations conferring INH resistance in genes coding for mycothiol biosynthesis (*mshA*, *mshC*, *mshD* in *M.smegmatis*) [55] and *aphC* [56] have also been suggested to reduce the capacity for pro-drug conversion, resulting in increased tolerance to INH. However these genes were not significantly differentially expressed in NRP2 relative to log phase bacilli. Most interestingly, recent evidence suggests that *M.tuberculosis* is able to maintain redox potential using the anabolism of propionate-derived lipids as a reductant sink [57]. A range of genes involved in fatty acid metabolism were differentially regulated in NRP2 relative to log phase (Supplementary Table 1). Furthermore, modulation of lipid metabolism in NRP2

may transform the architecture of the mycobacterial cell, which has been observed to thicken in stationary phase bacilli [58]. Changes to the cell wall structure affecting cell permeability (or the suppression of porin expression) may impact on a range of physiological processes [59]; not least in this scenario, limiting drug uptake that may result in increased tolerance to INH in non-replicating bacilli.

Both *fabG1* (*mabA*) and *inhA* were repressed in NRP2 compared to log phase growth; *inhA* was down-regulated 2-3 fold as determined by microarray and qRT-PCR. This reduction in INH target (*inhA*) is perhaps consequent on a redundant metabolic role for FASII in NRP2 due to the absence of cell wall synthesis in the non-replicating state. As such, INH does not induce a compensatory response in NRP2 as cell wall biosynthesis is already homeostatically down-regulated. Additionally, genes involved in mycolic acid modification *cmaA2*, *cmrA*, *mmaA2*, *mmaA3*, *mmaA4* and *umaA1* were also repressed in NRP2 relative to log phase bacilli. DNA replication encoding genes *dnaA*, *dnaB*, *dnaE1*, *dnaE2*, *dnaN*, *gyrA* and *ssb* [60] were down-regulated as expected in NRP2 relative to log phase growth, as were *recA*, *recF* and *recR*. The dihydrofolate reductase (product of *dfrA*), an essential precursor to DNA replication and postulated to be a target for INH [17], was also repressed in NRP2. It is therefore likely that it is the reduced requirement for mycolic acids and the FASII cycle, necessary for *de novo* cell wall biosynthesis, in NRP2 as replication ceases that confers phenotypic tolerance to INH.

**Use of transcriptional profiling to screen for NRP and INH-responsive genes that may influence *M.tuberculosis* drug tolerance**

Delineating the transcriptional response of *M.tuberculosis* to anti-microbial agents has lead to the characterization of mechanisms affecting drug tolerance. For example, the *iniB*/A/*C* genes, encoding an MDR-like pump, were identified to be induced after INH treatment in *M.tuberculosis* [61]. While deletion mutants were more sensitive to killing by INH; over-expression conferred resistance to both INH and ethambutol [62]. This transmembrane complex likely acts as an efflux pump maintaining cellular functions after the perturbation of cell wall biosynthesis. We reasoned that distinguishing genes that were differentially expressed in NRP2 that were also INH-responsive might reveal novel pathways that affect INH tolerance. We used the transcriptional program initiated by exposure to INH in log phase bacilli (in an INH-treated log phase *vs.* drug-free log phase comparison) to screen the 100s of genes differentially regulated in NRP2 relative to log phase bacilli (drug-free NRP2 *vs.* drug-free log phase) (Figure 5).

We isolated 23 genes (Box A) that were significantly induced by INH exposure that were also up-regulated in drug-free NRP2 relative to untreated log phase bacilli. 22 genes (Box B) repressed by INH were correspondingly repressed in NRP2 compared to log phase bacilli (Supplementary Table 4). 7 genes involved in fatty acid metabolism were induced in both NRP2 and INH-treated log phase bacilli (*fabG4*, *fadE5*, *fadA*, *fadB*, *prpC*, *fadE24* and *fadE23*) perhaps reflecting the redirection of lipid precursors, or metabolic shift to lipid degradation after environmental challenge. In addition, *fadD26*, *ppsA* and *ppsD*, part of the gene

cluster responsible for the biosynthesis of phthiocerol dimycocerosate, were induced in both scenarios. Over-expression of this propionate-derived complex lipid may modify the redox status of bacilli, and might also alter cell wall permeability, influencing INH tolerance. Furthermore, three genes that may also contribute to redox potential, *fprB*, an NADPH:aderenodoxin oxidoreductase (and the adjacent conserved hypothetical *Rv0885*), together with possible oxidoreducatase (*Rv1855c*), and putative monooxygenase (*Rv3049c*) were also induced. Other gene products that might influence drug kinetics such as the predicted membrane proteins of unknown function, *Rv0446c, Rv3346c*, *Rv3355c* and *Rv3675* were up-regulated in NRP2 and after INH exposure. Interestingly, *pknG*, encoding a protein kinase associated with the regulation of *M.tuberculosis* metabolism [63] and the ability to survive intracellularly [64], was induced by both INH and NRP2. The loss of *pknG* function has been previously implicated in the increased sensitivity of *M.smegmatis* to multiple antibiotics [65], perhaps due to a decrease in cell wall hydrophobicity. In addition, quantitative RT-PCR (Figure 4) revealed that *icl1* (encoding isocitrate lyase, part of the glyoxylate bypass), *narX* (involved in nitrate reduction), and *tgs1* (a triacylglycerol synthase) were all NRP2 and INH-responsive. These three genes have all been associated with the ability of *M.tuberculosis* to persist *in vivo* [66] [67] [42] [68].

## Conclusions

Understanding why drugs do and don't work during TB chemotherapy should advance novel drug design strategies, and reveal a little about the *in vivo* physiological states of *M.tuberculosis* bacilli that clearly influence the efficacy of antibiotic regimens. Transcriptional profiling is a powerful tool in this respect, mapping the expression pattern of all annotated genes in a whole-genome approach to defining the adaptations necessary for *M.tb* survival. This strategy does not however take into account processes affecting protein activity that also likely influence drug action. Here, we contrasted the *M.tb* transcriptional response to isoniazid in log phase and non-replicating bacilli (NRP2) as a model for INH tolerance. The Wayne model was selected to represent non-growing bacilli as this model is strictly defined, well characterized, and likely results in a largely homogenous *M.tuberculosis* population as bacilli display features of synchronous growth on exit from NRP2 [(31)]. Thus, a comparison between log phase, with the predominant gene expression signature derived from rapidly multiplying bacilli, and NRP2 bacilli should model the differences between INH-sensitive and INH-tolerant bacterial populations. Exposure of NRP2 bacilli to INH did not elicit a transcriptional response, and the RNA abundance of genes inducible by INH in log phase bacilli did not change after exposure of NRP2 bacilli to INH. This correlates with findings by Karakousis and colleagues who found the INH-mediated transcriptional program greatly diminished in oxygen and nutrient depleted *in vitro* models, and in murine lung tissue after 6 weeks aerosol infection [(34)].

Tolerance to INH in NRP2 bacilli cannot be correlated to the changing transcriptional profiles (comparing drug-free NRP2 to log phase bacilli) of genes directly implicated in INH resistance through resistance conferring mutations. However, it should be noted that factors affecting protein activity, such as the reduction of KatG catalase-peroxidase activity in hypoxic conditions [50], have not been characterized in this study. Phenotypic tolerance in NRP2 is likely the result of complex multi-gene adaptations to limited oxygen and diminishing carbon resources as bacterial multiplication slows and stops. The reduced requirement for mycolic acids and the FASII cycle in non-replicating bacilli renders bacilli tolerant to the effects of INH. Other factors such as redox state and cell wall permeability may also contribute to the abrogation of killing by INH in non-growing *M.tuberculosis* bacilli.

We speculate that the induction of a subset of genes involved in lipid metabolism and capable of performing oxidative functions in both NRP2 and after exposure to INH suggest that specific pathways remodelling the metabolic and respiratory state of *M.tuberculosis* may also influence the efficacy of INH killing. Furthermore, many of these genes have been identified to be up-regulated after macrophage infection [69] [70]. Could the induction of these pathways in replicating intracellular bacilli account for the significant decrease in INH sterilizing ability between axenic culture and after macrophage infection [71], or *in vivo* [72]? Finally, the up-regulation of *tgs1*, involved in triacylglycerol lipid body formation, after INH exposure and in NRP2 bacilli suggests that the metabolic consequences of specific growth constraints that induce a drug-

tolerant fat and lazy mycobacterial phenotype are also encountered in the human lung [42].

## Executive summary

1. Effective TB chemotherapy is hampered by prolonged treatment necessary to remove persistent bacilli, most likely because most front-line antibiotics primarily kill replicating bacilli.

2. An understanding of how and why *M.tuberculosis* bacilli become phenotypically tolerant to some antibiotics would illuminate current drug design programs, aimed at reducing duration of treatment from months to weeks.

3. Transcriptional profiling may be used to reveal important metabolic and physiological adaptations associated with *M.tuberculosis* infection *in vivo* and underpinning phenotypic tolerance to antibiotics. This genome-wide approach is also valuable in highlighting genes of interest that have no predicted function which may not have been identified by gene-specific assays. Novel insights into underlying mechanisms may thus be discovered.

4. *M.tuberculosis* bacilli in non-replicating persistence state 2 (NRP2) do not respond transcriptionally to INH exposure, and are tolerant to INH. Tolerance to INH in non-growing bacilli is therefore not a result of INH-inducible adaptive responses, but rather a reflection of the underlying metabolic state in which INH has minimal effect.

5. Altered transcript abundance of key genes implicated in INH resistance (involved in drug activation or target manipulation) do not explain why NRP2 bacilli become tolerant to INH.

6. The reduced requirements for mycolic acids and disparate redox state of non-growing bacilli probably result in tolerance to the effects of INH exposure.

7. Genes involved in lipid metabolism and alternative redox pathways may play functionally significant roles in INH-tolerant *M.tuberculosis* bacilli that persist through chemotherapy.

## Future perspective

There are several technological advances that impact on understanding drug resistance mechanisms, which together may resolve the important phenomenon of tolerance in *M.tuberculosis*.

1. The application of high-throughput whole genome DNA sequencing will transform *M.tuberculosis* resistance testing by mapping mutations to drug mode of action. Currently, in the absence of comprehensive, genome-wide correlation of genotypic changes with phenotypic drug resistance, it is difficult to predict resistance simply by sequence analysis for single nucleotide polymorphisms (SNPs). Implementation of, the soon to be available, rapid and cost effective third generation highly parallel sequencing platforms will permit every *M.tuberculosis* isolate to be sequenced. The correlation of genomic sequence to phenotypic resistance profile of many thousands of isolates will result in complex predictive modeling of resistance-modifying SNPs. However, it would be unwise to abandon sensitivity testing completely. This SNP mapping together with consequent gene-specific studies will expose multiple primary and secondary drug targets further elucidating mechanisms of antibiotic killing. It may also reveal new classes of mutation conferring low-level resistance, affecting features such as cell permeability or growth rate. Furthermore, such genome-wide analyses will begin to uncover linkage between resistance mutations, as well as a large number of mutations that have no effect on antibiotic resistance at all. The resulting payoff will provide clinicians with a resistance profile for problem isolates, enabling chemotherapy to be tailored effectively. Although tolerance is an adaptive process, genotypic changes

revealed by sequencing will define a panel of genes and highlight complex pathways that when modified at the mRNA or protein level may also influence antibiotic tolerance.

2. Predictive modeling of drug tolerance using transcriptional profiling and systems approaches to test *M.tuberculosis* drug-tolerant phenotypes will be instrumental in defining *in vitro* conditions that reflect the complex *in vivo* status. This will enable tolerance mechanisms to be investigated in models that accurately represent the antibiotic sensitivity profile of bacilli during natural infection, and allow drug-screening programs to target persistent-like bacilli. Modeling the changing physiological state of bacilli exposed to different *in vivo* or intracellular environments, diverse carbon sources and varied oxidative scenarios (defining mRNA, protein or metabolite levels) will help to delineate the unique transcriptional profiles derived from human sputa or lung tissue sections.

3. Single cell technologies and microfluidics have enabled bacterial populations to be visualized and differentiated; further advances in these fields will allow responses of individual bacteria to the changing microenvironment to be assayed. The identification and characterization of sub-populations of phenotypically diverse bacteria and the factors that influence their creation promises to reveal much about persisters, and their potential roles during infection. This, together with transcriptionally-defined drug-screening targeted to specific bacterial metabolic and respiratory states, should pave the way for discovery of novel compounds that kill non-replicating persistent bacteria. The continued support and patience of funding agencies will be required to smelt

these silver bullets with the hope of eliminating the hidden monster of persistent infection.

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## Financial disclosure and Acknowledgements

This work was supported by a postdoctoral fellowship to GT from the Ministerio de Educación y Ciencia (MEC), Spain (grant number 2005-0565) and the Spanish Network for Research in Infectious Diseases (REIPI, RD06/0008). PB would like to thank The Wellcome Trust for funding the Bacterial Microarray Group at St. George's (grant no. 062511). SW is supported by the European Commission 'New Medicines for TB - NM4TB' program (LHSP-CT-2005-018923). *M.tuberculosis* H37Rv reference DNA was kindly provided by Colorado State University (Contract No. HHSN266200400091C; NIH, NIAID N01-AI-40091).

The authors declare no conflicting financial interests.

## Figure legends

### Figure 1a

The transcriptional programs induced by isoniazid exposure in log phase, NRP1 and NRP2 growth states. The expression pattern of 71 genes induced by INH treatment in aerobic conditions are clustered alongside the transcriptional activity of these genes after INH exposure in NRP1 and NRP2. The genes are displayed as rows, growth conditions as columns. Asterisked genes were also INH-responsive in NRP1. No genes were significantly differentially expressed in NRP2 after INH treatment. Red coloring signifies induction; blue denotes repression relative to the respective drug-free growth state controls.

### Figure 1b

The diminished transcriptional response to INH exposure in NRP2. The gene expression profiles of 100 genes (71 induced, 29 repressed) significantly differentially expressed after INH treatment in aerobic conditions are plotted (-/+ INH) alongside the expression pattern of these genes in NRP1 and NRP2. The expression ratios are presented as fold change relative to drug-free growth state controls.

### Figure 2

The absence of a transcriptional response to INH in NRP2 bacilli, verified by quantitative RT-PCR with a panel of genes chosen to exemplify the INH transcriptional signature and the physiological status of bacilli. Expression ratios, marked in fold change, were calculated relative to *sigA* and drug-free growth controls using the ΔΔCt method. Standard deviations from triplicate

samples are marked. Red-pink coloring highlights genes selected to assay INH mechanism of action; blue coloring indicates genes chosen to illustrate the metabolic and respiratory state of bacilli.

**Figure 3**

The changes in RNA abundance of genes associated with INH resistance in log phase and NRP2 bacilli +/- INH relative to drug-free log phase bacilli. Expression ratios, derived from the microarray dataset, are plotted in fold change for log phase aerobic (AG) and NRP2 +/- INH treatment. Genes ordered by chromosome position, with gray bars highlighting adjacent genes. Annotations in black indicate that the over-expression of these gene products in NRP2 may be hypothesized to increase INH tolerance. Conversely, the repression of genes (marked in grey) might be expected to increase INH tolerance in NRP2.

**Figure 4**

Quantitative RT-PCR confirming the differential regulation of INH-responsive genes and genes indicative of bacterial metabolic state in log phase (AG) and NRP2 relative to drug-free log phase bacilli. Expression ratios +/- INH, measured in fold change, were calculated relative to *sigA* and untreated log phase control using the ΔΔCt method. Standard deviations from triplicate samples are marked.

**Figure 5**

The expression pattern of INH-responsive genes in log phase and NRP2 bacilli +/- INH relative to drug-free log phase bacilli. 100 genes, identified as significantly differentially regulated after INH exposure in aerobic conditions, were clustered alongside the NRP2 transcriptional profiles. Boxes A and B highlight genes either induced or repressed after both INH exposure and transition to NRP2 compared to untreated log phase bacilli. The genes are displayed as rows, growth conditions +/- INH as columns. Red coloring signifies induction; blue denotes repression relative to log phase drug-free control. Asterisked genes were significantly differentially expressed in both drug-free NRP2 and INH-treated aerobic bacilli compared to untreated log phase bacilli.

**Supplementary Table S1**

Genes significantly differentially regulated in NRP2 compared to log phase bacilli, ttest $p<0.05$, Benjamini and Hochberg multiple testing correction, >1.5 fold change. Ordered by chromosome position.

**Supplementary Table S2**

Genes significantly differentially expressed after isoniazid treatment of log phase bacilli relative to drug-free control, $p<0.05$, Benjamini and Hochberg multiple testing correction, >1.5 fold change. Ordered by chromosome position.

**Supplementary Table S3**

Primers and probe sequences (5'-3') for the panel of qRT-PCR assays used in this study.

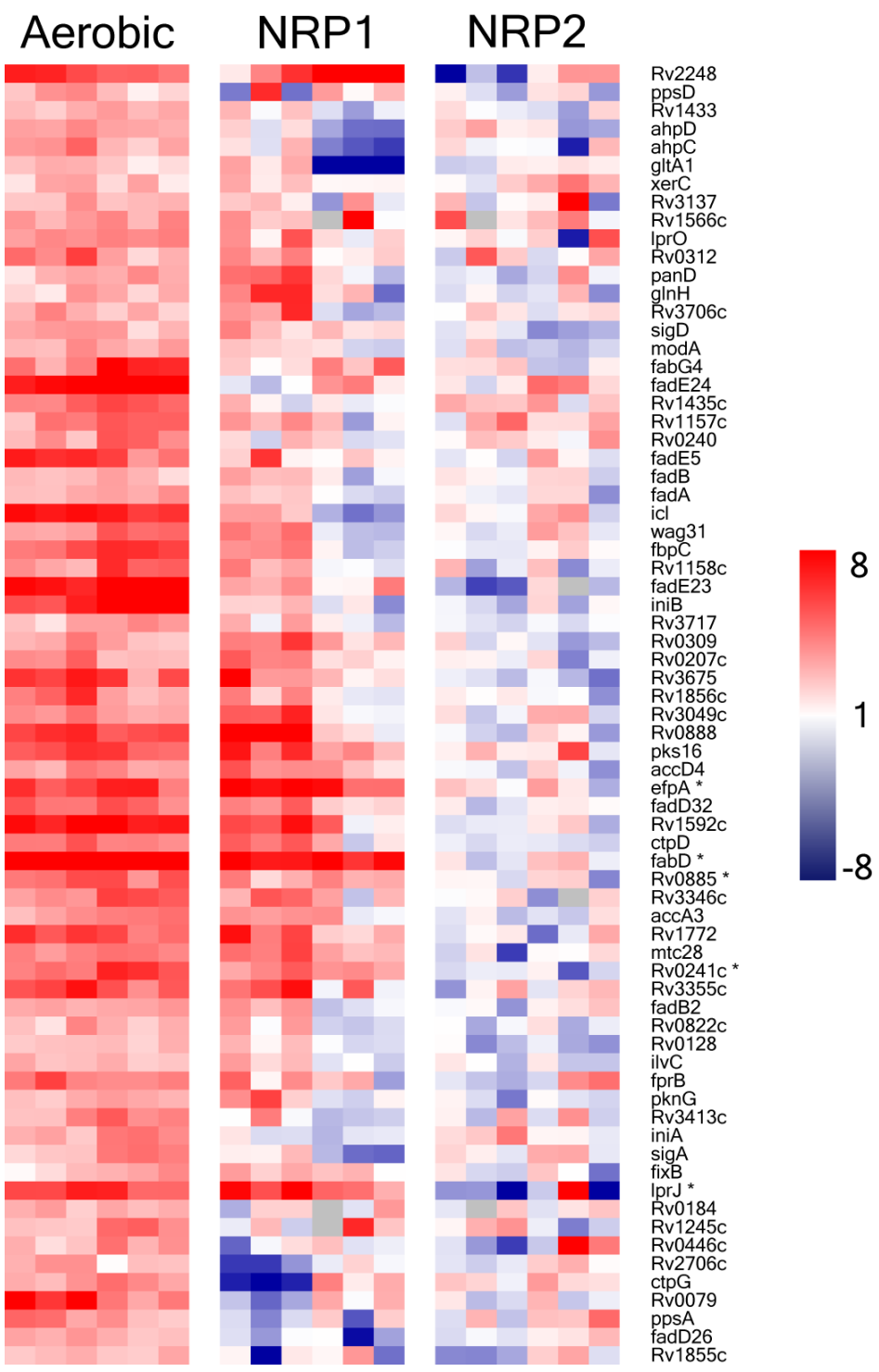
**Supplementary Table S4**

Figure 5 Boxes A and B. Genes identified to be both INH-responsive and differentially regulated in NRP2 relative to log phase bacilli. Ordered by chromosome position.

**Supplementary Figure S1**

Growth of *M.tuberculosis* in aerobic conditions (plotted in red), and in non-replicating persistence stage 1 (NRP1, green) and NRP2 (blue). Vertical arrows indicate isoniazid exposure and RNA extraction time intervals at day 3 (aerobic), day 6 (NRP1) and day 21 (NRP2). The standard deviations between replicate experiments are marked as error bars.

**Figure 1a**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NRP2
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ppsD
Rv1433
ahpD
ahpC
gltA1
xerC
Rv3137
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lprO
Rv0312
panD
glnH
Rv3706c
sigD
modA
fabG4
fadE24
Rv1435c
Rv1157c
Rv0240
fadE5
fadB
fadA
icl
wag31
fbpC
Rv1158c
fadE23
iniB
Rv3717
Rv0309
Rv0207c
Rv3675
Rv1856c
Rv3049c
Rv0888
pks16
accD4
efpA *
fadD32
Rv1592c
ctpD
fabD *
Rv0885 *
Rv3346c
accA3
Rv1772
mtc28
Rv0241c *
Rv3355c
fadB2
Rv0822c
Rv0128
ilvC
fprB
pknG
Rv3413c
iniA
sigA
fixB
lprJ *
Rv0184
Rv1245c
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fadD26
Rv1855c
8
1
-8

**Figure 1b**

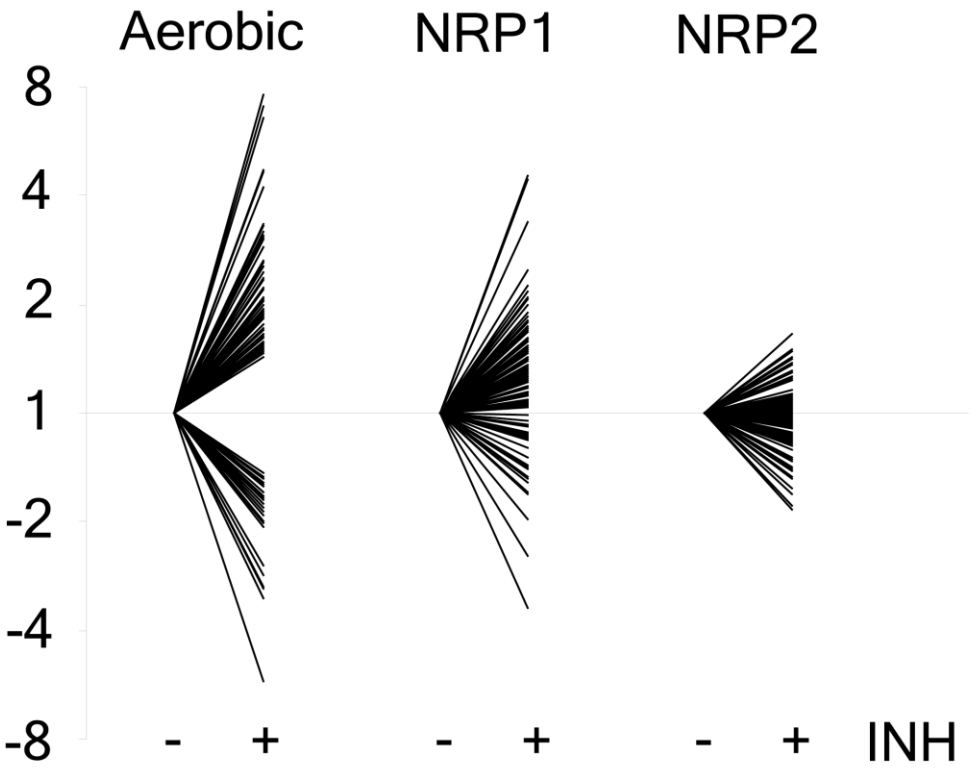



**Figure 2**

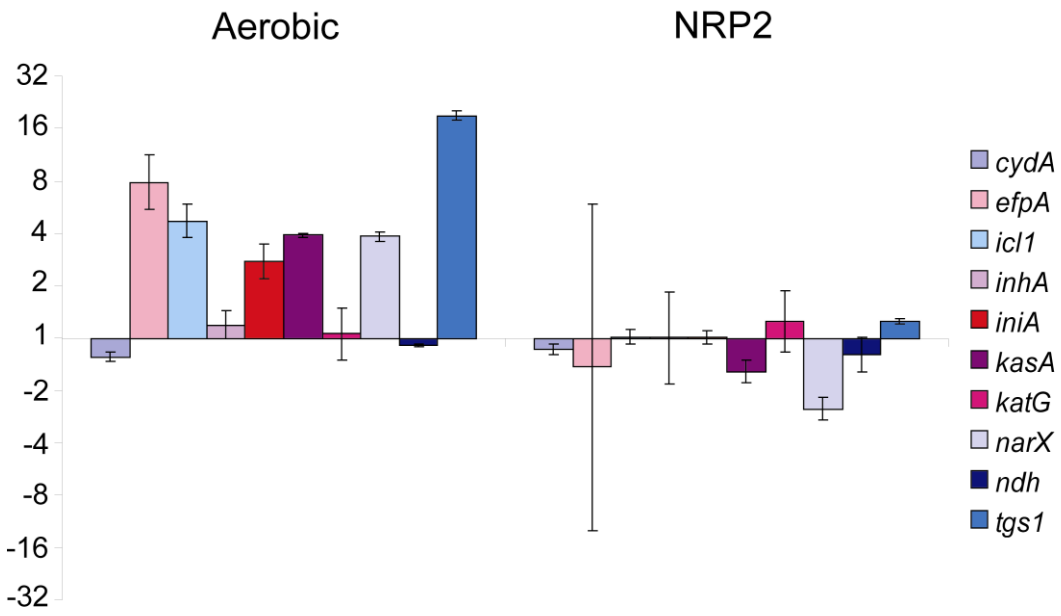


**Figure 3**

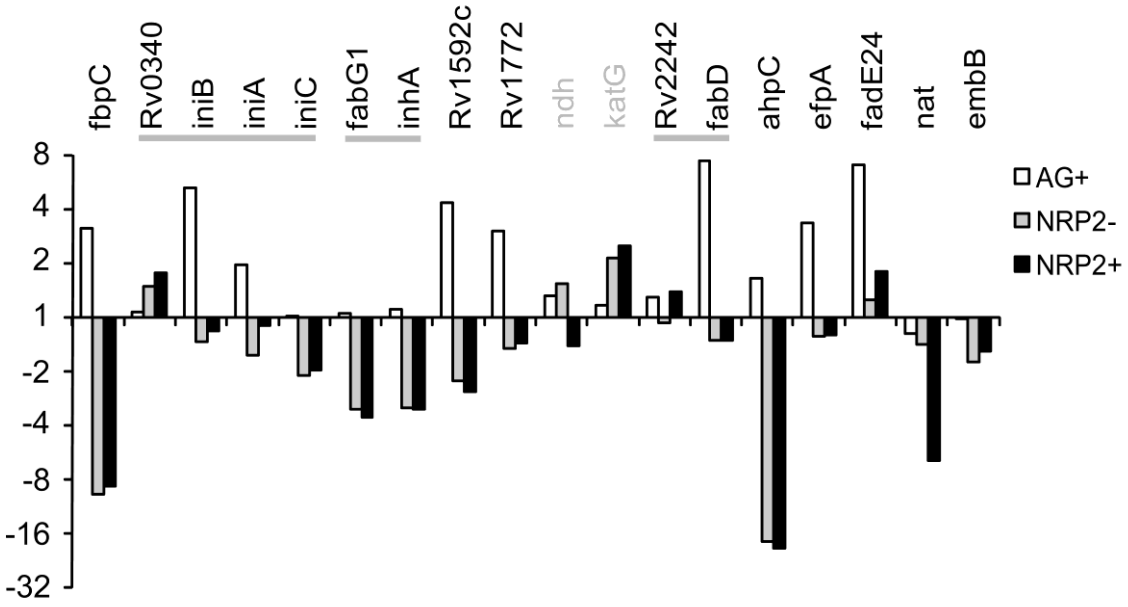



**Figure 4**

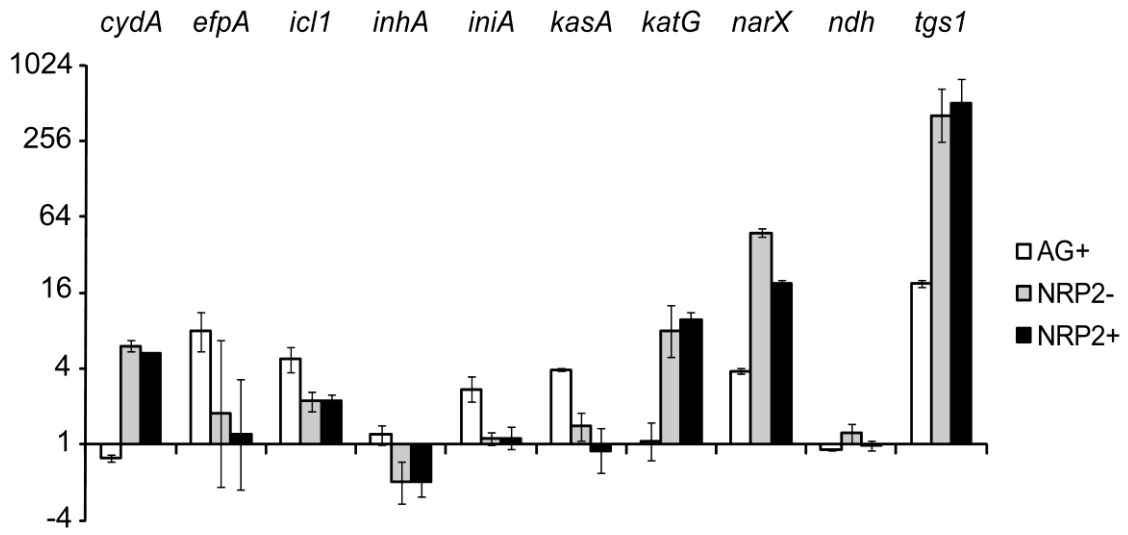
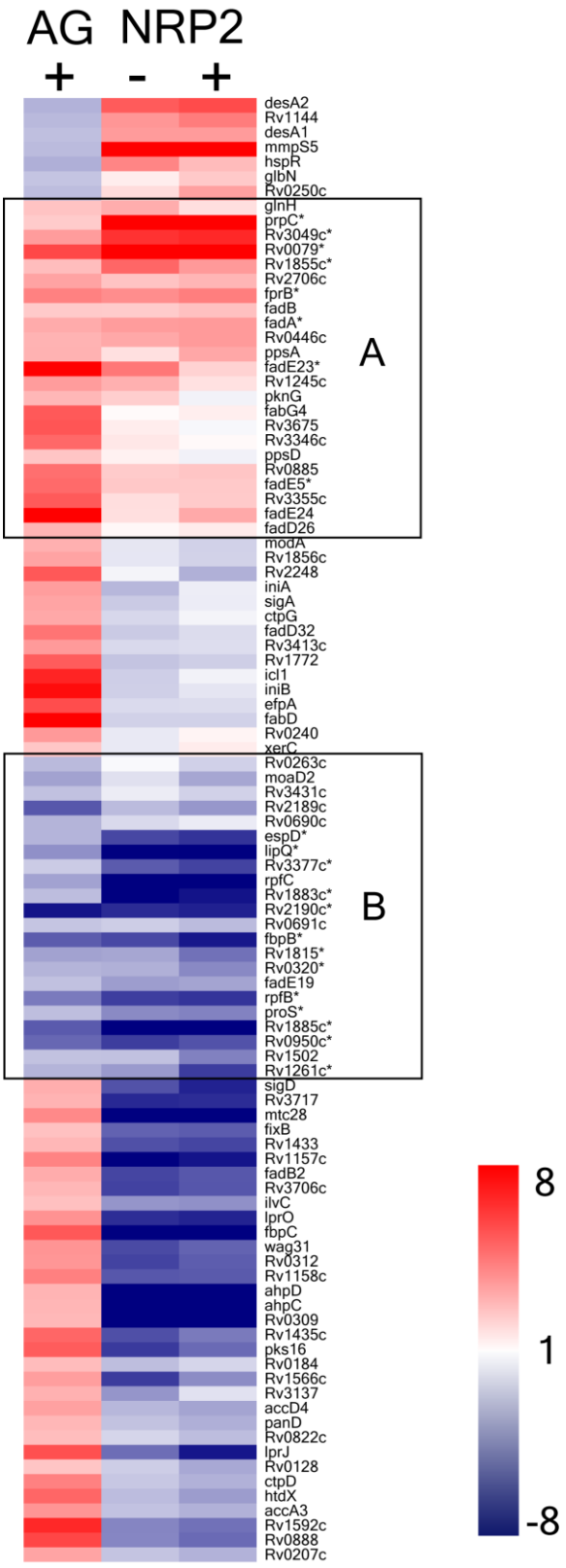


**Figure 5**


AG NRP2
+ - +
desA2
Rv1144
desA1
mmpS5
hspR
glbN
Rv0250c
glnH
prpC*
Rv3049c*
Rv0079*
Rv1855c*
Rv2706c
fprB*
fadB
fadA*
Rv0446c
ppsA
fadE23*
Rv1245c
pknG
fabG4
Rv3675
Rv3346c
ppsD
Rv0885
fadE5*
Rv3355c
fadE24
fadD26
A
modA
Rv1856c
Rv2248
iniA
sigA
ctpG
fadD32
Rv3413c
Rv1772
icl1
iniB
efpA
fabD
Rv0240
xerC
Rv0263c
moaD2
Rv3431c
Rv2189c
Rv0690c
espD*
lipQ*
Rv3377c*
rpfC
Rv1883c*
Rv2190c*
Rv0691c
fbpB*
Rv1815*
Rv0320*
fadE19
rpfB*
proS*
Rv1885c*
Rv0950c*
Rv1502
Rv1261c*
B
sigD
Rv3717
mtc28
fixB
Rv1433
Rv1157c
fadB2
Rv3706c
ilvC
lprO
fbpC
wag31
Rv0312
Rv1158c
ahpD
ahpC
Rv0309
Rv1435c
pks16
Rv0184
Rv1566c
Rv3137
accD4
panD
Rv0822c
lprJ
Rv0128
ctpD
htdX
accA3
Rv1592c
Rv0888
Rv0207c
8
1
-8

**Supplementary Figure 1**